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ORIGINAL ARTICLE

Identification of mutations in Iranian patients' DAX-1 gene with X-linked adrenal hypoplasia congenital

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KEYWORDS

X-linked adrenal hypoplasia congenital (X-linked AHC);
DAX1 protein;
NR0B1 gene

Abstract *Objective(s):* X-linked adrenal hypoplasia congenital (X-linked AHC) is a rare disorder, characterized by infantile-onset acute primary adrenal insufficiency and hypogonadotropic hypogonadism (HH) at an average age of three weeks and onset in roughly 40% is in childhood. Its cause is an inactivating mutation in the (nuclear receptor subfamily 0, group B, member 1) NR0B1 gene, DSS (dosage sensitive sex)-AHC vital region on the X-gene 1.

Subjects and methods: In the present study, the (dosage-sensitive, sex reversal, adrenal hypoplasia congenital, important region on the X-chromosome, gene 1) DAX-1 gene from four Iranian patients with X-linked AHC was analyzed by means of polymerase chain reaction (PCR) and direct sequencing.

Results: We identified a polymorphism (Rs6150) which encodes a cysteine (Cys) at position 38, a de novo deletion, c.849-928del79 bp, c.849-856ins, (TGCTGCA) mutation and a missense mutation, Leu262Gln, which encodes a leucine (Leu) for glutamine (Gln) at position 262.

Conclusion: Both mentioned mutations are located at crucial and functional region DAX1 protein. They are detected in the C-terminal region of DAX1 protein which is involved by the conserved amino acid chain as well as transcriptional silencing domain. By considering other investigation, mutations in this region probably lead to produce a misfolded protein. Consequently, the misfolded protein would not work influentially in order to inhibit some gene expression. As a result, our findings will expand the variety of DAX1 mutations. On the other hand, it is revealed that these mutations play a key role in the pathogenesis of AHC, thus, recognizing these new mutations will facilitate the patients prognosis producer as well as raising the clinical knowledge about this rare disease.

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1. Introduction

Congenital adrenal hypoplasia (AHC) is a rare cause of congenital adrenal insufficiency and was first described by Sikl [1]. It is estimated to affect 1 in 12,500 newborns [2]. X-linked adrenal hypoplasia congenital (X-linked AHC) is an inherited disorder of adrenal gland development, characterized by absence or near absence of the permanent zone of the adrenal cortex [3]. AHC is mostly present in infancy or early childhood with primary adrenal failure with symptoms of salt-wasting, hyperpigmentation, hyponatremia, hyperkalemia, reduction of serum glucocorticoid and aldosterone and increase in plasma (adrenocorticotropic hormone) ACTH and affected boys typically have hypogonadotropic hypogonadism (HH) at the time of puberty [3,4]. Overall, careful clinical management of the affected children is important, because rapid and life-threatening deterioration of adrenal function frequently follows an asymptomatic period during infancy [5]. Thus, early diagnosis ensures an early start of mineralocorticoid and glucocorticoid treatment and prevents sudden death.

The responsible gene for AHC is DAX1 which encodes a protein that is a member of the orphan nuclear hormone receptor superfamily [6]. It is suggested that mutations in DAX1 (also called NROB1) gene in chromosome Xp21 are responsible for the X-linked AHC [7]. The DAX1 gene is composed of two exons separated by a single intron and encodes a 470 amino acid [3,8]. Moreover, Ligand binding domain is localized, similar carboxyl terminus of DAX1 protein to other nuclear receptors, but lacks the typical zinc finger DNA-binding domain in the amino terminus. As an alternative, the amino terminus consists of 3.5 alanine/glycine-rich repeats of a 65–70 amino acid motif involved in protein–protein interactions and may bind to hairpin loop structure in DNA [2,9]. Previously, DAX1 expression has been shown in the developing adrenal cortex, gonad, anterior pituitary, and hypothalamus and also in the adult adrenal cortex, anterior pituitary, the hypothalamic ventromedial nucleus, Sertoli and Leydig cells in the testis, as well as theca and granulose cells in the ovary [2,10]. DAX1 plays a principal role as a transcriptional repressor in the gonadal and adrenal development and in the regulation of gonadotropin production [11].

The aim of this study was to characterize clinically and genetically four Iranian patients with X-linked AHC plus analyzing their NR0B1 gene.

Table 1 Clinical characteristics in four patients with X-linked AHC.

Case	Diagnosis age of disease	Initial symptoms	Family history	Mental retardation
1	2 years	Vomiting generalized pigmentation failure to thrive	–	–
2	3 years	Vomiting Poor feeding Decrease in consciousness	–	–
3-1	3 months	Vomiting Poor feeding	+	–
3-2	50 days	Vomiting Poor feeding failure to thrive Diarrhea	+	–

2. Subjects and methods

2.1. Patients

This clinical trial was performed in Imam Reza Hospital related to Mashhad Medical University, Mashhad (Iran) in 2013. The study was approved by local Ethics Committee and also financially supported by the research vice chancellor of the University. Informed consent was taken from all cases parents and the study was approved by it. After obtaining approval and written consent, 4 children who were diagnosed with AHC and confirmed by a pediatric physician were selected to investigate their mutation.

Clinical symptoms in patients and laboratory findings respectively are presented in Tables 1 and 2. All of the cases are under treatment with Hydrocortisone and Fludrocortisone and also 100 mg of Testosterone, monthly. They did not show any secondary sex characteristics when they were 14 year old. Overall, by considering their clinical histories and test results, the diagnosis of X-linked AHC was considered and

Table 2 First laboratory findings.

Cases	Na[a]	K[b]	Urea[c]	Creatinine[d]	ACTH[e]	Cortisol[f]	Cortisol with short acting test	17-Hydroxyprogesterone[g]
1	117	6.5	26	0.7	1050	3.5	3.6	60
2	108	6.2	75	0.6	1200	1.5	1.7	48
3-1	119	4.6	30	0.7	1105	2	2.2	54
3-2	128	6.1	10	0.8	800	1.5	1.8	56

[a] Normal range for Neonate (132–147) mEq/L. Children (135–145) mEq/L.
[b] Normal range for Neonate (3.5–6.1) mEq/L. Children (3.5–5.1) mEq/L.
[c] Normal range (5–18) mg/dl.
[d] Normal range (0.6–1.3) mg/dl.
[e] Normal range (7.2–63.3) pg/ml.
[f] Normal range in the morning (5–23) mcg/dl. In the afternoon (3–16) mcg/dl.
[g] Normal range for prepubertal male <110 ng/dl.

Table 3 Laboratory findings at 14.5 years cases.

	LH[a]	FSH[b]	T[c]	CPK[d]	Aldolase[e]	ACTH[f]	Na[g]	K[h]
1	0.12	0.13	< 10	86	4.36	789	139	4.1
2	0.6	0.9	< 10	73	5.2	70	142	4.3
3-1	0.45	1.8	< 10	47	6.53	315	143	4.2
3-2	0.5	1.4	< 10	143	9.2	> 810	139	3.9

[a] Normal range for tanner stage I: (0.04–3.6), St II: (0.2–4.8), St III: (0.5–6.3), St IV and V: (0.56–7.8) mlu/m.
[b] Normal range for stage I: < 1.3, St II: (1–7), St III: (2–9), St IV: (2–10), St V: (3–12) mlu/ml.
[c] Normal range for stage I: (2–23), St II: (5–70), St III: 15–210), St IV: (86–401), St V: (200–685) ng/dl.
[d] Normal range (40–225) U/L.
[e] Normal range(Up to 7.6) IU/L.
[f] Normal range (7.2–63.3) pg/ml.
[g] Normal range for adults (135–145) mEq/L.
[h] Normal range for adults (3.5–5.5) mEq/L.

Table 4 Sequence of primers.

Primer	Sequence	Product length (bp)	Annealing temperature (°C)
DAX1.For Ex1.F1	TGAGACAGGGAAAGGGGTAAT	423	53.9
DAX1.Rev Ex1.F1	CCGGGCTCATCGCCGCACGAA	423	72.4
DAX1.For Ex1.F2	TGGTGGATCAGTGTTGGGGC	241	60.0
DAX1.Rev Ex1.F2	CCGGGATCAGAGCCGCACGAA	241	67.9
DAX1.For Ex1.F3	AAGCAAACGTACGCGGCAC	279	58.3
DAX1.Rev Ex1.F3	CCTCTGCGCGAAGTAGGAGC	279	58.7
DAX1.For Ex1.F4	TAGCTCAAAGCAAACGCACGTG	426	59.9
DAX1.Rev Ex1.F4	GACGCCCAGCAGTTGCGCAC	426	65.6
DAX1.For Ex1.F5	GCCTCAGCGGGCCTGTTGAAG	450	64.7
DAX1.Rev Ex1.F5	CCCGATGCTTTTGTGAGCTGGGAA	450	66.7
DAX1.For Ex2.F6	GCTAGCAAAGGACTCTGTGGT	321	52.5
DAX1.Rev Ex2.F6	TGTGTGGCCCACATGACTTTA	321	55.9

consequently, they were administered to our genetic investigation (see Table 3).

2.2. Polymerase chain reaction (PCR) and direct sequencing of the DAX-1 gene

DNA was extracted from patients' blood leukocytes using the standard method of salting out. Both exons of DAX1 were PCR amplified using six primer pairs, presented in the Table 4. These primers are designed based on published article "Mutational Analysis of DAX1 in Patients with Hypogonadotropic Hypogonadism or Pubertal Delay". They are synthesized by Takapouzist biological company, Tehran, Iran.

PCR was optimized by varying annealing temperature and primer ratios. Briefly, the optimized condition was performed in a mixture of 2 μL DNA 1 μL forward primer and reverse primer, 8.5 μL distilled water with 12.5 μL master mix of red 2× Amplicon.

The PCR profile was as follows: initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for1 min, the optimized primer annealing at 58 °C–65 °C for the 30 s–1 min and extension at 72 °C for 5 min. PCR reactions were performed using TECHNE Applied Biosystems Thermocycler and PCR productions were sent to Harvard University for direct sequencing.

3. Result

3.1. PCR-direct sequencing of the DAX-1 gene

In 4 Iranian patients, one missense mutation (L262Q) in two brothers and a novel mutation c.849-928del79 bp, c.849-856ins (TGCTGCA) in one case and an Rs6150 polymorphism were identified. In case 1, Rs6150 polymorphism, was due to a transition of C to T at nucleotide 114, that both of them encoding cysteine (Cys) at position 38 was identified (Figs. 1 and 2). In case 2, novel deletion mutation was identified. This mutation had a deletion of 79 bases at positions 283 to 310 with the insertion of 7 nucleotides (TGCTGCA) (Figs. 3–7). Only the patient's brother has not shown any symptom of the disease. His mother was analyzed for mutation of NR0B1 gene, but no mutations were identified. However, 3-1 and 3-2 patients have same parents, it is revealed that both of them are carrying missense mutation L262Q (c.785 T > A). This mutation was due to a transversion of T to A at nucleotide 785, which encodes a leucine (Leu) for glutamine (Gln) at position 262 (Figs. 8 and 9).

4. Discussion

The association between DAX-1 and X-linked AHC is well-established, and many mutations in DAX-1 have been depicted

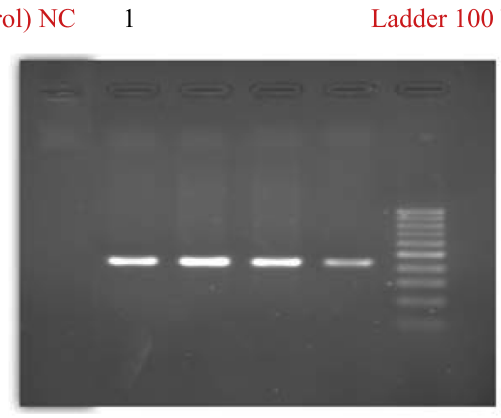

Figure 1 Electrophoresis of the PCR-amplified productions of F1 (first fragment of exon1) in agarose gel (2%).

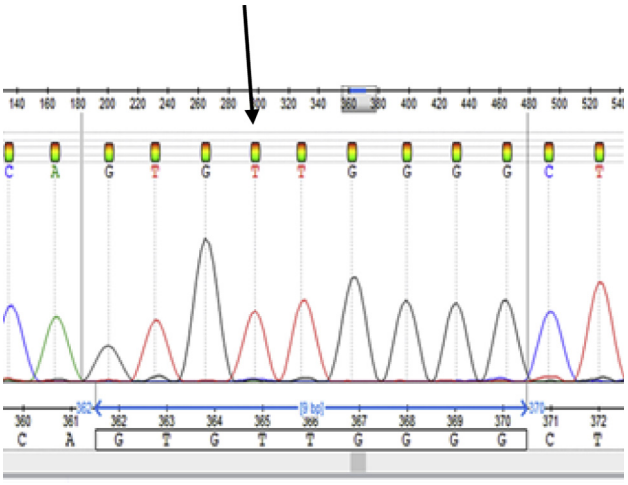

Figure 2 Partial nucleotide sequence of the DAX-1 gene, exon 1, fragment 1 of case 1, for Rs6150 polymorphism.

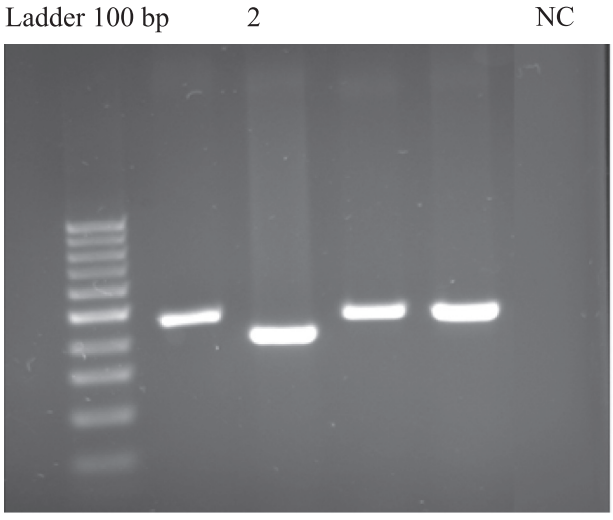

Figure 3 Electrophoresis of the PCR-amplified productions of F5 (fifth fragment of exon1) in agarose gel (2%).

in patients with this condition. In the present study, we identified two mutations, including one missense mutation, one de novo deletion and insertion mutation of the DAX-1 gene in patients with X-linked AHC and also we identified one polymorphism. The current identified missense mutation of c.785 T > A is encoding a leucine (Leu) for glutamine (Gln) at position 262 was detected in two brothers. These patients had repeated vomiting strikingly in addition case 3-2 had more severe symptoms than case 3-1. Interestingly, family history demonstrates that 2 infants had died for repeated vomiting. Similarly as Muscatelli et al. (1994) reported that adrenal insufficiency typically presents acutely in male infants with vomiting, feeding difficulty, dehydration, and shock caused by a salt-wasting episode [6]. In addition, Bae et al. (1996) declared that missense mutations may be important for identifying critical regions or amino acids necessary for DAX-1 function [12]. Actually, DAX1 as an orphan receptor belonging to the superfamily has the N-terminal region of DAX1 is considered to serve as a DNA-binding domain and the C-terminal region contains a putative ligand-binding domain [2]. Comparably, most missense mutations of the DAX-1 gene are located in the C-terminal presumptive ligand-binding domain [12]. The Leu262Gln mutation is located in the C-terminal presumptive ligand binding (Fig. 10). Generally, all DAX-1 missense mutations reported to date affect amino acids that are conserved completely in all species in which a DAX-1 homolog has been cloned, even including the chicken and alligator [13,14]. This finding shows the importance of a C-terminal region that conserved amino acids may play a functional role for Dax1 protein. DAX-1 missense mutations affect amino acids that form the hydrophobic core of the DAX-1 (ligand bonding domain) LBD. These amino acids may affect subdomain structure rather than ligand binding and may be involved in ligand-independent interactions such as dimerization [15]. It is also possible that the amino-terminus of DAX-1 is not important for its function. More likely, the repeated nature of the domains within the amino-terminus may serve redundant functions, such that single amino acid substitutions are not sufficient to cause complete loss of function. The transcriptional silencing domain of DAX1 has been mapped to a bipartite region in the C-terminal LBD-like (LBD-L) domain which includes two groups of residues, one at each end of the LBD-L [16]. Interestingly, all documented AHC missense mutations also map to this region [15], and it has been shown that AHC mutations abolish transcriptional silencing activity, suggesting that a lack

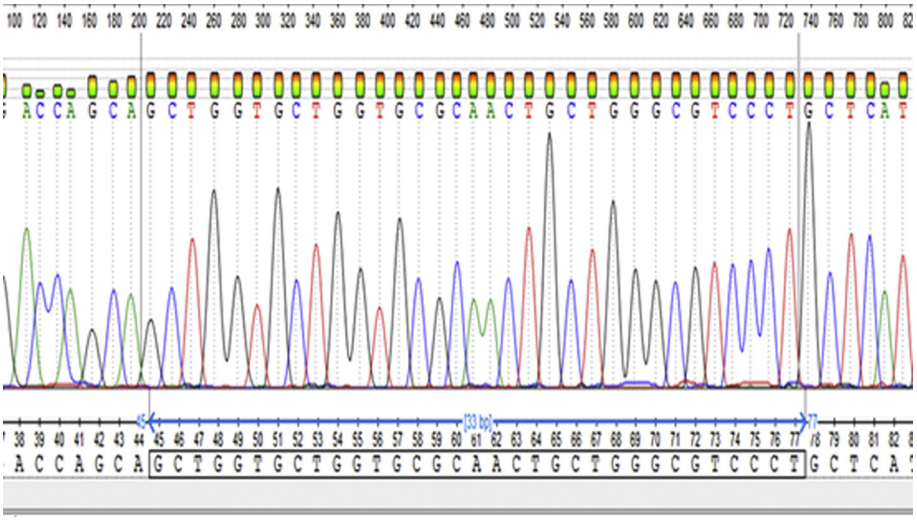

Figure 4 Forward partial nucleotide sequences of the DAX-1 gene, exon 1, fragment 5 of a normal case.

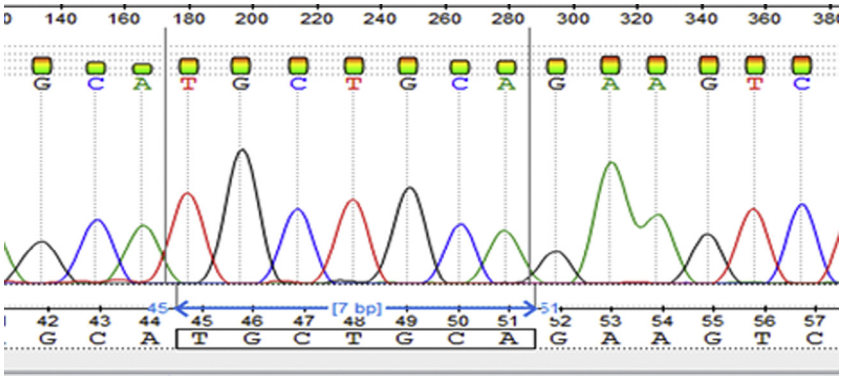

Figure 5 Forward partial nucleotide sequences of the DAX-1 gene, exon 1, fragment 5 of case 2 for c.849-928del79 bp, c.849-856ins (TGCTGCA) mutation.

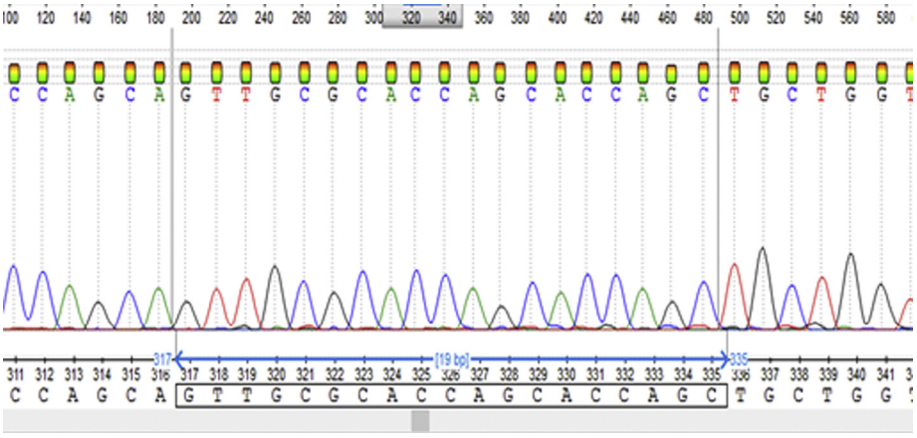

Figure 6 Reverse partial nucleotide sequences of the DAX-1 gene, exon 1, fragment 5 of a normal case.

of silencing could be involved in the pathogenesis of AHC. In spite of all that more studies are needed to identifying the effect of L262Q on DAX1 protein and pathogenesis of AHC.

A de novo deletion mutation is a rare event and a de novo deletion c. 849-928del79 bp, c.849-856ins (TGCTGCA) of the DAX-1 gene has not previously been reported. Identification of a case of a de novo deletion mutation of the DAX-1 gene reinforces the importance of molecular analysis of the patient's mother for genetic counseling. The mother of case 2 told us that she didn't have another affected or carrier child. Although we

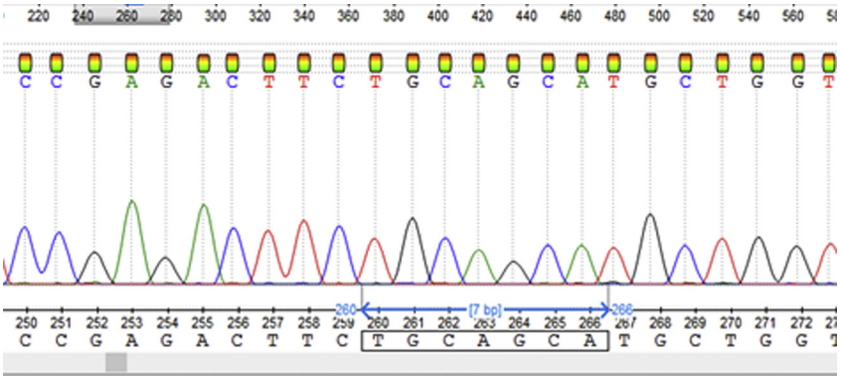

Figure 7 Reverse partial nucleotide sequences of the DAX-1 gene, exon 1, fragment 5 of case 2 for c.849-928del79 bp, c.849-856ins (TGCTGCA) mutation.

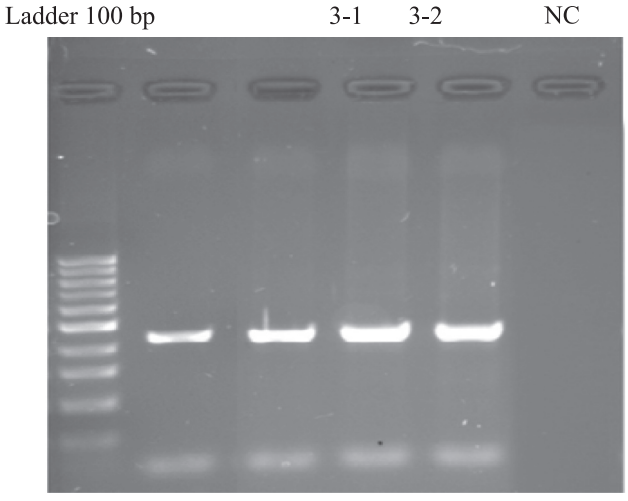

Figure 8 Electrophoresis of the PCR-amplified productions of F4 (fourth fragment of exon 1) in agarose gel (2%).

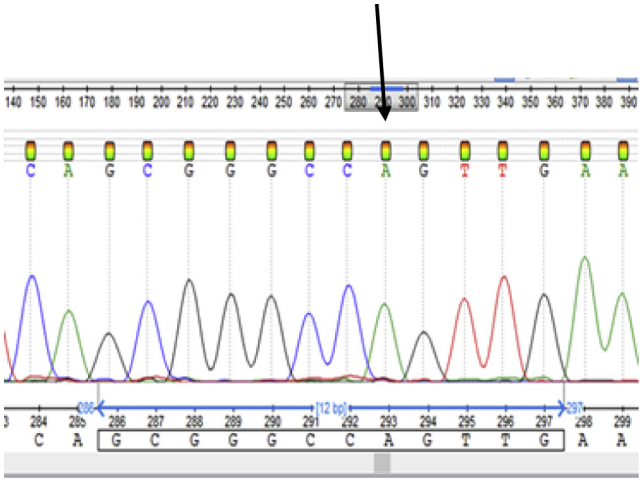

Figure 9 Partial nucleotide sequence of the DAX-1 gene, exon 1, fragment 4 of case 3-1 and 3-2 for c.785 T > Amutation.

cannot totally exclude the possibility that gonadal mosaicism does exist for DAX-1 mutations, patients with X-linked AHC due to gonadal mosaicism have not been reported. Indeed, the evidence that a mother and elder brother of case 2 have

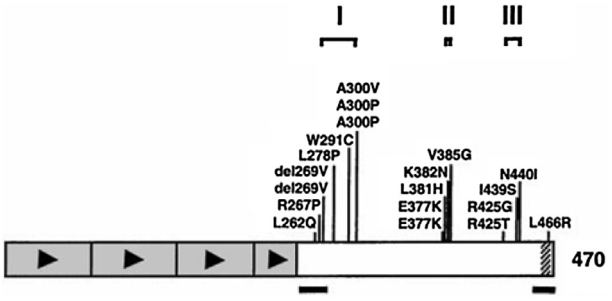

Figure 10 Naturally occurring DAX-1 missense mutations cluster within the carboxyl-terminus of DAX-1 in a region homologous to the LBD of other nuclear receptors. The repeat motif structure of the amino-terminus of DAX-1 is shown by arrows. The carboxyl-terminal putative AF2 domain is shaded. The carboxyl-terminal putative AF2 domain is shaded. Previously reported potential transcriptional silencing domains are shown by black bars [17].

no deletion of the DAX-1 gene may exclude the possibility of gonadal mosaicism in this family. This deletion occurred in C-terminal of DAX1 protein. The majority of functional data reported suggesting that DAX-1 represses the transcription of several genes expressed in the adrenal and reproductive axes, either directly or through an interaction with the related orphan nuclear receptor steroidogenic factor-1 (SF-1) [16]. Some studies have suggested that this repressor function may involve specific transcriptional silencing domains within the carboxyl-terminus of DAX-1 [17] whereas others have proposed that DAX-1 can bind directly to hairpin loop structures in the promoters of certain target genes [9]. Further, direct interactions between DAX-1 and repressors such as nuclear receptor and corepressor (NCoR) [18] and Alien [19] have been described. However, the relative importance of these mechanisms in the regulation of different target genes remains unclear. This deletion may cause misfolding DAX1 protein and will not be able to interact with corepressors so, will not have correct interaction with ligands such as SF1 (Fig. 11). Indeed, loss of as little as 9 amino acids at the carboxyl-terminus of DAX-1 is sufficient to impair DAX-1 function and to produce a clinically severe phenotype [20].

In the Li et al. survey some missense and deletion mutations in the C-terminal region of DAX1 protein were identified.

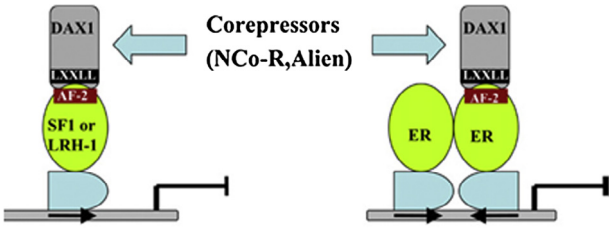

Figure 11 Mechanisms of DAX1-mediated repression of SF1, ER, and LRH-1. DAX1 binds the AF-2 domain of the nuclear receptors via its LXXLL motifs and recruits corepressor proteins to target gene promoters [2].

Through this study, the transcriptional activities of the identified mutations were assessed in vitro and showed loss of repression of SF-1-mediated (steroidogenic acute regulatory protein) StAR and (luteinizing hormone beta-subunit) LHβ expression. Furthermore, they found that DAX1 mutation resulted in a reduction of (Gonadotrophin-releasing hormone) GnRH expression, which is associated with HHG [21]. In spite of all this the study suggested more investigation into identifying the effect of this novel mutation on DAX1 protein structure, function and pathogenesis of AHC is needed.

We also identified one synonymous polymorphism c.114C.T (rs6150) in NR0B1 gene in a patient. This polymorphism is located within the first exon, and neither changes the predicted amino acid. Even though this DNA change has not been examined experimentally for their effect on mRNA or protein expression, Phelan and McCabe, 2001 stated that this nucleotide change does not co-segregate with the disease phenotype which confirms that this is a true polymorphism, through repeated observations by different groups [22]. Conversely, it is interesting that while case 1 had more severe and obvious symptoms for AHC disease, no mutations were found for it and just the c.114C.T (rs6150) polymorphism is identified. This study suggests more investigation is needed for correlation between c.114C.T (rs6150) and AHC disease.

In conclusion, two different mutations are characterized in the C-terminal domain of DAX-1 including a missense mutation and a de novo deletion of the DAX-1 gene. These mutations lead to disruption in the ligand binding domain. Thus, the normal biological activity of the DAX-1 protein, such as adrenal embryogenesis and/or hypothalamic-pituitary activity may influence these mutations. On the other hand, the identification of a novel de novo deletion of the DAX-1 gene reinforces the importance of family genetic counseling for a patient with X-linked AHC and HH. As a result, our findings will expand the number of DAX1 mutations reported in the literature, however, it will highlight the role of these mutations the pathogenesis of AHC. Finally, all data will facilitate the patients' prognosis procedure as well as increase clinical knowledge of this rare disease.

Conflict of interest

None.

Acknowledgment

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